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Use and limitations of light microscopy for diagnosing malaria at the primary health care level

D. PAYNE[1]

Recent developments in diagnostic techniques for malaria, particularly DNA probes and sero-immunology, have raised questions as to how these techniques might be used to facilitate malaria diagnosis at the most peripheral levels of the primary health care system. At present, malaria diagnosis is based on the light microscope and is likely to remain so in the immediate future. This article describes how the diagnosis of malaria by light microscopy has been improved over the years, and expresses the need for further improvement while concomitant research into other standardized and simplified techniques for the diagnosis of malaria is vigorously pursued.

The discovery by Laveran in November 1880 of parasites ("éléments parasitaires") in the red blood cells of a malaria patient was made by light microscopy and, since then, no other diagnostic means has superseded the combination of the human eye and the light microscope for the routine diagnosis of human malaria.

This update reviews:

— how techniques were refined and modified over the past century so that physicians and other health workers at the peripheral level of health care could detect the malaria parasite in blood samples;
— the advantages and limitations of these techniques and how new technology may change the situation.

DETECTION OF PARASITES

The compound microscope

The size of the human malaria parasite ranges from a diminutive 1 μm (early trophozoite) in *Plasmodium falciparum* to 15 μm or more in *P. vivax* or *P. ovale*; the larger gametocytes of *P. falciparum* only rarely exceed 10 μm in the longer axis. Accordingly considerable magnification is required to see them in the necessary detail for differential diagnosis, the ideal range being somewhere between 500 and 1000× total magnification.

All modern laboratory microscopes meet the above minimum total magnification and most of them even the above maximum, using a combination of Huygenian eyepieces (5–10× magnification) and high-power objectives—usually oil-immersion—of 50 to 100× magnification. Some of the eyepieces have widefield capabilities which increase the scanning area by 25% or more, while the objectives are almost uniformly corrected for chromatic aberration, an important consideration when dealing with the vital diagnostic nuances of differential stains.

For the routine microscopical diagnosis of malaria a total magnification of between 600 and 700× provides the ideal compromise between enhancement in size and retention of fine detail. Many standard microscope lens systems are approaching their practical limits at 1000× magnification and the relative increase in object size is more than offset by reduced definition and enhanced colour aberration. These limitations are further exacerbated by widefield eyepieces which have been shown in comparative trials to be inferior to the standard lens system in parasite detection, especially when parasite densities are low.[a]

Unfortunately many manufacturers adhere to a now almost standard format of 10× widefield eyepieces and a 100× oil-immersion objective, presumably because of public demand based on the misconception that a higher magnification is always better.

The illumination system

Efficient malaria microscopy is impossible without adequate illumination. Unfortunately, this aspect of

[1] Malaria Action Programme and UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, World Health Organization, 1211 Geneva 27, Switzerland. Requests for reprints should be addressed to the Malaria Action Programme.

[a] STOREY, J. ET AL. *Field research project in epidemiology and control of malaria in African savannah, Kano, Nigeria.* Unpublished WHO document, MPD/TN/73.1, Technical notes No. 7–15 (1973).

laboratory management rarely receives the attention it requires. The problem has been exacerbated in recent years by the wholly desirable and increasing trend towards binocular microscopes which, however, need at least double the illumination of the monoculars they replaced. This problem has been further aggravated by the tendency (mentioned earlier) of manufacturers to increase the effective working magnification of their binocular microscopes by providing, as standard, 10× widefield eyepieces. Unfortunately, the writer knows of no microscope system which will provide adequate illumination for a binocular microscope with a total magnification of 1000× using a daylight illumination system. Indeed, only microscopists with acute visual sensitivity can obtain adequate illumination at this magnification using the standard 220-volt tungsten bulb system, which is provided with most standard compound binocular microscopes in use today in peripheral health units. Moreover, in order to achieve this marginal level of illumination it is necessary to set the light augmentation rheostat (when provided) at close to its highest level. This not only greatly shortens the bulb's life, but enhances the evaporation of the tungsten element which condenses on the inner surface of the bulb and in time further reduces the effective illumination.

The microscope slide and blood films

The definitive diagnosis of malaria has historically been based on the detection of the parasite in the blood. Initially this was achieved by observing the parasites in the red blood cells in thin blood films, the recognition of the parasites being enhanced by the use of Romanowsky-type differential stains which selectively colour the nuclear and cytoplasmic material. Despite the relative inefficiency of thin blood film examinations—Dowling & Shute found that it would require 30 times as long to find a parasite in a thin film as in a thick film of the same patient (*1*)—the thin blood film still retains its popularity as a diagnostic technique primarily in hospitals and clinics.

It has long been recognized (*2*) that a much more efficient screening technique is that of the thick blood film, a dehaemoglobinized layer of blood cells which is about 20–30 times denser than the thin film. The comparative loss of parasite diagnostic characteristics in the thick film, which the disruption of the host cell (during dehaemoglobinization) entails, is not a serious encumbrance to the experienced microscopist, the more professional of whom anyway always insist on an accompanying thin film for definitive diagnostic purposes in the rare event that this cannot be resolved in the thick film.

The standard basis of examination of a thick film is usually 100 microscope fields. In a good, standard thick film this is equivalent to approximately 0.25 μl blood (Dowling & Shute (*1*) reported a mean volume of 0.23 μl). Accordingly, this gives a theoretical parasite detection threshold of four or more parasites per microlitre of blood and this is considered quite adequate for routine malaria diagnostic purposes.

Efforts aimed at improving the rate of parasite detection in the thick film by centrifugation have not proved successful, principally owing to staining problems (*3–5*).

Staining of blood films

As mentioned earlier, the detection of malaria parasites in the blood has been routinely facilitated by differential stains which have the property of staining the nuclear material red and the cytoplasmic material blue. This reaction only takes place under ideal conditions of pH. Theoretically this should be neutral (pH 7.0), with the basic and acidic ions in balance. However, owing to the optical dominance of red pigmentation, an optically perfect result is obtained at the slightly more basic pH of 7.2.

Of the many differential stains that have been developed over the years the aqueous Romanowsky stains (discovered in 1891) have proved to be the most adaptable and reliable for routine work. While Leishman's stain (1901) undoubtedly gives the most exquisite results in the thin film, Giemsa stain (1902) has proved to be the best all-round stain for the routine diagnosis of malaria. It has the disadvantage of being relatively expensive, but this is outweighed by its stability over time and its consistent staining quality over a wide range of temperatures. Even in the hands of a novice it will produce consistently good and lasting staining and in the hands of the expert it is incomparable. It is the stain of choice for peripheral health laboratories.

Alternative microscopical techniques[b]

Various detection methods other than simple differential stains have been employed over the years without notable success. More recently, Shute & Sodeman (*6*) and Richards et al. (*7*) described techniques using acridine orange staining and fluorescence microscopy. However, although acridine orange staining might have an advantage when large

[b] Developments in electronic image analysers, which theoretically could be programmed to identify some forms of malaria parasite, are not considered here as the costs (most existing equipment exceeds US$ 100 000 per unit) rule out their use at the peripheral health care level, even if a practical application could be devised.

malaria parasites were present, it was inferior to Romanowsky staining for the detection of young trophozoites, the difference becoming more pronounced as the parasite concentration decreased.

Dark-field microscopy was used in another approach to detect malaria parasites by fluorescent-antibody staining of blood films (*8*). However, the methodology described was complex, requiring the use of raised inverted coverslips and, of course, specialized equipment for dark-field microscopy. Its value as a reliable diagnostic tool at the peripheral health unit level has yet to be evaluated.

QUANTIFICATION AND LIMITATIONS

Enumeration of parasites

The knowledge of the density of an infection of malaria has always been a useful adjunct to the diagnosis of the species as this often, but not always, serves as a useful guide to the severity of the infection, its probable duration, and possible evolution. This is particularly so in life-threatening infections of *P. falciparum*. However, in recent years the introduction of various enumerative techniques has enabled the standard thick and thin films to be used to monitor, *in vitro* and *in vivo*, the development of resistance to antimalarial drugs and thus serve as an important information base in the formulation of appropriate drug policies on the national and international level.

The nature of thin and thick films imposes definite limits on the elements which can be used in enumerative techniques. Essentially, since the films are dried, no perception is possible of the former volume of the constituents of the blood unless a measured volume of blood is placed onto a predetermined area of the blood slide. Even here, at the microscopic level, there is no certainty that the blood cells will be evenly distributed, and to ensure that truly comparable volumes of blood were examined, it would be necessary to examine all, or at least a significant proportion, of the calibrated blood film.

Such an approach—fixed volume in a pre-established area—was developed over 50 years ago by Earle & Perez and used in the Americas (*9*). Three much more widely employed parameters are:

—the percentage of infected red blood cells (only possible in the thin film);
—the number of parasites per white blood cell; and
—the number of parasites per microscope field.

All these indicators can be transformed into an index of parasites per volume of blood by assuming a constant number of red or white blood cells or of microscope fields in that volume, i.e., 5 000 000 erythrocytes or 8000 leukocytes or 400 microscope fields per microlitre of blood.

While at first glance these indices appear crude—particularly given the wide variation in blood cells, which occurs naturally and in association with disease, and the variation in individual thick films—these differences are in fact not very important in real terms: the number of the blood cells rarely varies by more than a factor of 1 either way. Accordingly, with this variation, a count of four malaria parasites per microlitre of blood could be in error to the extent of really being two parasites or eight parasites per microlitre of blood. This difference is not of significance to the physician or the epidemiologist. Equally, of course, parasitaemias stated as being 200 000 or 800 000 instead of being the actual 400 000 per microlitre are similarly accurate enough for diagnostic, treatment and monitoring purposes.

It is the common practice in hospitals and clinics to express malaria parasitaemias as a percentage of the red blood cells infected, while enumeration for epidemiological or research purposes is usually given as so many parasites per 10 000 erythrocytes (thin films only) or per microlitre of blood.

In either event the counting is usually done as a separate activity after species identification has been made. To ensure that mixed and low-grade infections are not missed, it is usual to examine a thick blood film for 100 fields, which is equivalent to 2000 leukocytes (approximately 0.25 μl blood) or about six minutes of examination time. Unless infections are heavy, counting techniques using thin films are not applicable for the routine diagnosis of malaria.

Once the diagnosis has been established, a separate procedure for reckoning the parasite density is undertaken. A convenient and widely accepted method uses hand-tally counters (1 to 9999) or counting machines whereby successive representative fields of a thick blood film are examined and the totals of two, or more, numerators (e.g., leukocytes and asexual malaria parasites) are cumulatively totalled. Thresholds are established to minimize the counting to a level of reasonable significance: 500 asexual parasites or 1000 leukocytes, whichever is counted first. While this appears relatively crude theoretically, it will detect parasitaemias as low as eight parasites per microlitre of blood, which is quite adequate for most enumeration requirements.

The literature describes various attempts over the years to rationalize and define the reliability of microscopic diagnosis of malaria in the field and the laboratory. Raghavan tried a purely statistical approach with special reference to the efficacy of cross-checking procedures in malaria eradication programmes (*10*). He found that thick film examinations using the

standard 100-field criterion could only detect with "reasonable certainty" (i.e., 99% probability) parasite densities of 44 parasites in 1000 thick film fields, i.e., 4.4 parasites per 100 thick film microscope fields. Unfortunately, he did not state the parameters which define his "standard" thick film.

Dowling & Shute avoided these ambiguities by carefully calculating the average thickness of the thick films and thin films they used in a comparative study (*1*). Their conclusions were that while a high proportion of parasites are lost during staining, this loss being more significant in scanty infections, a parasite count as low as one per mm^3 could be detected in a thick film examination of ten minutes' duration. While this is not equated to the number of fields examined, it is broadly in line with the detection threshold given earlier of four or more parasites in six minutes of thick film examination time.

Limitations in the efficacy of microscopical diagnosis

The establishment and maintenance of a reliable and efficient diagnostic facility for malaria at the primary health care level is dependent on many (or a combination of) factors, which can seriously degrade the utility and relevance of the malaria laboratory. These can be broadly summarized as follows:

(*a*) *Deficiencies in personnel*: inadequate training; poor supervision; bad management including the improper utilization of the available expertise and work time.

(*b*) *Substandard or inappropriate equipment*: incorrect or poorly coordinated specifications, particularly of microscopes; irregular maintenance and replacement of worn-out parts; poor quality control of stains and reagents; inappropriate supply schedules which either cause delays in the supply of material or accumulate stocks which exceed the shelf-life of the material.

(*c*) *Inappropriate technology*: improper use of techniques (e.g., thin films for routine malaria diagnosis) or the introduction of new technology which in use is less efficient at the peripheral level than well-established methods; continuation of programmes and routines which were once relevant but do not meet the current needs of primary health care.

(*d*) *Organizational deficiencies*: the maintenance of an efficient and relevant organizational structure calls for frequent review and analysis of the current aims and objectives of the malaria diagnostic facility as well as the necessary changes in staffing and methods to meet these requirements with the resources available. From time to time this may involve radically changing how personnel and other resources are utilized and overcoming the inertia to change at both local and higher levels.

CONCLUSIONS AND RECOMMENDATIONS

(1) In the development of new diagnostic techniques for malaria, emphasis should be placed on simple and standardized technology as well as low cost so that they can be used even in the periphery of the primary health care system and provide the best possible guidance for the treatment of malaria in endemic areas. It is at present unlikely that there will be any dramatic technical discovery or development that will in the immediate future render the light microscope entirely obsolete at the primary health care (PHC) level for routine laboratory examinations of various kinds. The almost universal use and versatility of the light microscope would make the adoption of any other methods problematic unless they could be shown to be simple, reliable, and inexpensive. A species-specific sero-immunological diagnostic test for malaria based on colorimetric changes on filter-paper could probably meet these criteria and receive widescale adoption at the PHC level. However, an indication of the cost barrier at this level is the current limited application of the simple colorimetric tests now available for glucose-6-phosphate-dehydrogenase (G-6-PD) determinations which, though only costing US$ 1 per test, have not been adopted generally despite a widely-accepted need for such monitoring programmes in the affected populations.

(2) Since it is accepted that the light microscope still has an important role to play in the diagnosis of malaria, efforts should be made to ensure that the equipment and materials provided are appropriate for the work to be undertaken and that their specifications accurately reflect the real requirements and potentialities of the laboratory at the PHC unit where they are to be utilized.

In general terms the specifications are as follows:

(*a*) *Microscopes*: binocular vision; magnifications in the range of 600–700× based on a 100× oil-immersion spring-loaded lens and appropriate Huygenian eyepieces (widefield eyepieces are an unnecessary additional expense); adequate illumination systems (interchangeable for daylight and artificial light) for the maximum total magnification of the microscope; standardized construction to facilitate maintenence and stocking of spare parts.

(*b*) *Stains*: aqueous Romanowsky stains, preferably Giemsa (with proper quality control if formulated locally).

(*c*) *Enumeration apparatus*: hand-held tally counters or simple digital electronic counters.

(3) The enumeration of parasite density is an extremely useful adjunct to species diagnosis and of great importance in epidemiological studies. Since

the examinations carried out at the PHC level generally provide much of the statistical data on malaria generated in a country, the more detailed the information that is routinely collected, the more meaningful and useful the statistics are.

(4) Laboratory staff at the PHC level with a sound background in light microscopy technology are an extremely useful resource when special studies, such as *in vivo* and *in vitro* studies in malaria, are required in a particular area.

RÉSUMÉ

EMPLOI ET LIMITES DE LA MICROSCOPIE OPTIQUE POUR LE DIAGNOSTIC DU PALUDISME AU NIVEAU DES SOINS DE SANTÉ PRIMAIRES

Depuis la découverte des plasmodies en 1880, le microscope optique a couramment servi au diagnostic de routine du paludisme. Même aujourd'hui, le diagnostic repose sur l'identification de l'une quelconque des quatre espèces de *Plasmodium* parasites de l'homme dans des frottis sanguins colorés observés au microscope à immersion dans l'huile. Le dénombrement des plasmodies et l'identification de leurs stades de développement dans le sang se font également couramment par ce moyen.

Les microscopes modernes sont en général de type binoculaire composé et sont équipés d'un objectif à immersion dans l'huile donnant un grossissement de l'ordre de 500× à 1000×. Cependant, le grossissement optimal pour le diagnostic du paludisme avec un microscope de laboratoire classique est de 600× à 700×, et donne une bonne définition ainsi qu'un minimum d'aberrations chromatiques, mais au niveau des soins de santé périphériques, de nombreux microscopes sont équipés d'oculaires à grand champ et grossissent de 800 à 1000 fois. Ces grossissements inutilement élevés exigent souvent un éclairage dépassant les possibilités du système utilisé, et dans de nombreux cas, les microscopes sont utilisés en routine avec un éclairage insuffisant, qu'il provienne d'une source naturelle ou artificielle.

On peut identifier les plasmodies sur des étalements de sang épais ou minces: de nombreux hôpitaux et dispensaires utilisent encore les étalements minces alors qu'ils demandent une durée d'observation 30 fois plus longue. La coloration de ces étalements est d'une importance capitale si l'on veut tirer le meilleur parti des possibilités de coloration différentielle. Le Giemsa s'est révélé convenir le mieux à l'utilisation sur le terrain et, à condition d'ajuster correctement le pH, donne la plupart du temps des résultats uniformes.

Les autres techniques microscopiques de diagnostic, basées sur la centrifugation du sang et sur d'autres types de coloration, ne se sont encore jamais révélées plus efficaces en pratique. Il n'est pas possible d'appliquer les techniques de dénombrement des plasmodies en fonction d'un volume de sang donné, mais on peut obtenir des estimations acceptables de la parasitémie en employant d'autres indicateurs, tels que le nombre de plasmodies par rapport au nombre d'hématies ou de leucocytes, ou par champ microscopique, dans des étalements épais et minces classiques. Les limites de l'efficacité du diagnostic microscopique du paludisme en routine au niveau des soins de santé primaires sont en grande partie dues à: *a*) un personnel non compétent car mal formé et mal encadré; *b*) l'utilisation d'un matériel de qualité médiocre ou inadapté; et *c*) une mauvaise organisation.

Il est peu probable qu'on puisse dans un avenir proche mettre au point des techniques diagnostiques qui remplaceront totalement le microscope optique pour le diagnostic de routine du paludisme au niveau des soins de santé primaires, car les obstacles techniques et financiers sont considérables. Par conséquent, mieux vaut s'attacher à optimiser l'emploi des méthodes et techniques actuelles pour assurer un diagnostic paludologique sûr au moyen du microscope optique, jusqu'à ce qu'on puisse disposer d'une autre technique qui se soit révélée d'un usage satisfaisant au niveau du système des soins de santé primaires.

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